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Effect of Methyl Farnesoate on Late Larval Development and Metamorphosis in the Prawn *Macrobrachium rosenbergii* (Decapoda, Palaemonidae): A Juvenoid-like Effect?

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Abstract. Methyl farnesoate (MF), the unepoxidated form of insect juvenile hormone III, was detected in larvae of the freshwater prawn *Macrobrachium rosenbergii*, which metamorphose to post-larvae following 11 larval stages. The possible role of MF as a morphogen was studied by administering the compound to *M. rosenbergii* larvae *via* an *Artemia* vector. Higher MF levels caused earlier retardation of late larval growth, and the highest dose retarded larval development. Furthermore, MF significantly affected the patterns of metamorphosis and the appearance of intermediate individuals exhibiting both larval and post-larval morphology and behavior. Three intermediate types were defined, two of which were found only at the MF-treated groups and one that was exclusive to the higher dose treatments. The relative abundance of intermediate specimens increased from 2% in the control to 32% in the high MF concentration, which suggests that MF has a juvenoid-like effect in this decapod crustacean.

Introduction

Juvenile hormones (JHs)—a family of epoxidated sesquiterpenoids—act as growth regulators in insects (Wigglesworth, 1970). Methyl farnesoate (MF), the unepoxidated form of JH III, has been detected in some insect species (Slama, 1971; Lanzrein *et al.*, 1984; Bruning *et al.*, 1985), but its physiological significance is not clear. The first report of the presence of MF in Crustacea was that of Laufer *et al.* (1987), who demonstrated that MF is secreted by the mandibular organ of the spider crab *Libinia emarginata* and is found in its hemolymph. Since then, MF has been found in a variety of crustaceans (Borst *et al.*, 1987; Laufer and Borst, 1988; Landau *et al.*, 1989), including the freshwater prawn *Macrobrachium rosenbergii* (Sagi *et al.*, 1991).

JHs are involved in the regulation of a substantial number of insect functions, including development, reproduction, and behavior. JHs are probably best known for their effect on the growth and differentiation of insect epidermal cells. When JHs are present during larval instars, the cuticle that is secreted has larval characteristics; in the absence of JHs, the cuticle exhibits pupal characteristics (see Laufer and Borst, 1983, for a review). Exogenous application of JHs may cause the appearance of various intermediate forms, and in some cases, of supernumerary larvae, as a result of differences in the growth rates of various parts of the body (allometric growth) (reviewed in Sehnal, 1983). It has been suggested that exogenous JH applied during a critical period may function at the cellular level, resulting in mosaic animals with characteristics of two different stages (see Riddiford, 1994, for a review). Thus, it is likely that the effect of a particular juvenoid on insect development is determined by the developmental timing and the effective concentration of the juvenoid (Slama, 1995).

The regulation of larval development and metamorpho-

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Abbreviations: MF, methyl farnesoate; JH, juvenile hormone.

sis in crustaceans is not fully understood. In cyprids of barnacles, JH I and JH analogs caused premature metamorphosis without attachment of the cyprid to the substrate (Gomez *et al.*, 1973; Ramenofsky *et al.*, 1974; Tighe-Ford, 1977). In decapods, JH analogs retarded the development of larvae of the mud crab *Rhithropanopeus harrisii* (Christiansen *et al.*, 1977a, b) and the growth of early larval stages and post-larvae of the estuarine shrimp *Palaemonetes pugio*. In contrast, growth was enhanced in the premetamorphic stages of *P. pugio* (McKenney and Celestial, 1993). Treatment of *Homarus americanus* larvae with JH III increased the duration of larval development and influenced the size of the carapace and appendages (Hertz and Chang, 1986). Injections of JH I into *H. americanus* larvae gave rise to a number of intermediate stages (Charmantier *et al.*, 1988).

MF is the only JH-like compound found thus far in crustaceans. A few studies have targeted its regulatory role in development and metamorphosis in decapods. Application of MF to *H. americanus* larvae prolonged metamorphosis by a small but significant amount of time (Borst *et al.*, 1987). In *M. rosenbergii*, MF retarded growth and development in larval stages 5 to 9 (Abdu *et al.*, 1998).

In some crustaceans the processes of larval development and metamorphosis are similar to those in hemimetabolous insects (Snyder and Chang, 1986). Such is the case for *M. rosenbergii*, which exhibits a "common type" of larval development (Sollaud, 1923), encompassing 11 stages, followed by metamorphosis into post-larvae (Uno and Soo, 1969). Larval development in *M. rosenbergii* is characterized by significant growth of the carapace from stage 1 through stage 11. During metamorphosis (from stage 11 to post-larva), growth is allometric, with increases in size being limited to certain parts of the body (excluding the carapace) (Abdu, 1996).

In this study, *M. rosenbergii* was used as a model species for studying the role of MF in the late larval development and metamorphosis of crustaceans. MF was indeed detected in the larvae, and its effect on the manifestation of morphological features during metamorphosis was studied.

Materials and Methods

Detection of methyl farnesoate in Macrobrachium rosenbergii *larvae*

M. rosenbergii larvae were hatched and reared according to Daniels *et al.* (1992). Larval stages were determined according to Uno and Soo (1969). To detect the presence of MF, about 1 g of larvae at each of the stages 4, 6, 9, 10, and 11, and post-larvae were collected and homogenized in 2 ml of 4% NaCl and 5 ml of acetonitrile. The homogenates were filtered through α-cellulose, washed with 4% NaCl and acetonitrile, and extracted with hexane. The hexane fraction was evaporated by means of a rotary evaporator. The dry matter was dissolved in hexane and passed through a Sep-Pak silica cartridge (Waters, Milford, MA), and the cartridge was rinsed with methylene chloride (Abdu *et al.*, 1998). The methylene chloride was evaporated, and the residue was dissolved in 100 μl of hexane, loaded into a Beckman HPLC and analyzed according to Sagi *et al.* (1991). The nonbiological isomer, *cis-trans* MF, was used as the internal standard, and the retention times were compared to an external standard containing both the biological (*all-trans* MF) and nonbiological isomers. The putative *trans-trans* MF peaks of larvae at stages 4, 6, 9, 10, and 11 were collected from the HPLC and loaded onto a Hewlett Packard gas chromatograph with a 30-m cross-linked and surface-bound dimethylpolysiloxane column (0.5 min to 70°C, 70°–280°C, 20°C/min) connected to a mass spectrometer.

Effect of methyl farnesoate on late larval growth and development

Three experimental groups, each containing 15 *M. rosenbergii* stage-9 larvae, were fed with *Artemia* enriched with 0.21, 0.35, or 0.59 μg MF/ml according to Abdu *et al.* (1998). A control group was fed with *Artemia* enriched with the vehicle alone (ethyl alcohol and SUPER-SELCO). Each treatment was performed in three replicates. The experimental system consisted of 12 dark plastic containers (300 ml) that were constantly aerated. The water was replaced daily, and the temperature was maintained at 27° ± 1°C. The larvae were fed *ad libitum* with *Artemia* nauplii that were enriched daily with MF. Five larvae per replicate were randomly sampled twice a week: larval stage was determined and the length of the carapace was measured with an ocular micrometer; the larvae were then returned to the growth chamber. In the calculation of the average larval stage for each sampling, all post-11 stages (including both post-larvae and intermediate individuals) were designated as 12. The average larval stage and the average carapace length were compared between treatments, and the statistical significance was tested using one-way ANOVA followed by the least significant difference (LSD) test $P \leq 0.05$. The experiment was repeated three times with similar results; each experiment contained 180 larvae.

Assessment of metamorphosis in Macrobrachium rosenbergii

Among the many changes occurring during metamorphosis (Ling, 1969; Uno and Soo, 1969), the following six predominant changes were selected to distinguish between larvae and post-larvae of *M. rosenbergii* in this study: (1) In larvae, the abdomen is bent such that the

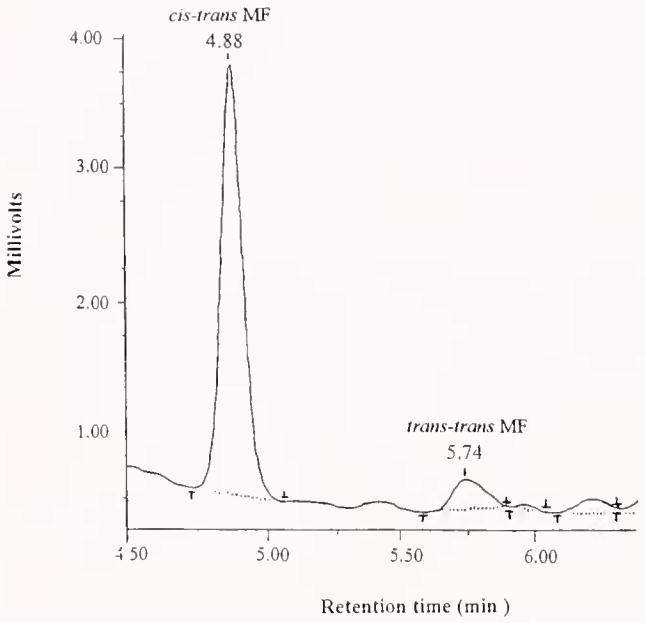

Figure 1. HPLC detection of methyl farnesoate in *Macrobrachium rosenbergii* larvae at stage 4. *Cis-trans* methyl farnesoate was used as the internal standard.

pleopods and the walking legs are not in the same plane, whereas in post-larvae, the abdomen is straight, with the pleopods and the walking legs in the same plane. (2) Larvae swim mainly by beating the long exopodites of the pereiopods. During the metamorphic process these long exopodites degenerate and become reduced to knobs at the postmetamorphic molt (Ling, 1969). (3) In stage 11 larvae, the endopodite of the second pereiopod has three segments, the second and third segments being at an angle to the first, which is not fully developed. In contrast, in post-larvae there are four segments, all of which are straight, and the first segment is fully developed, as in the adult. (4) The carapace median tooth is evident from larval stage 4 onward, but disappears during metamorphosis and is absent in the post-larva. (5) In stage 11 larvae, the entire dorsal margin of the rostrum is equipped with numerous small teeth, but no ventral teeth are present, whereas the rostrum of the post-larva has 11 large dorsal teeth and 5 ventral teeth. (6) Larvae are planktonic, swimming tail up, and in most cases tail first, ventral side up; during metamorphosis, they settle to the bottom. Post-larvae are benthic, swimming and behaving as do the juveniles.

The relative abundance of the different intermediate types were compared between treatments, and the statistical significance was tested using the Mann-Whitney *U* test.

Results

Presence of methyl farnesoate in Macrobrachium rosenbergii *larvae*

MF was found in hexane extracts of *M. rosenbergii* larvae at stages 4, 6, 9, 10, and 11, and in post-larvae. A

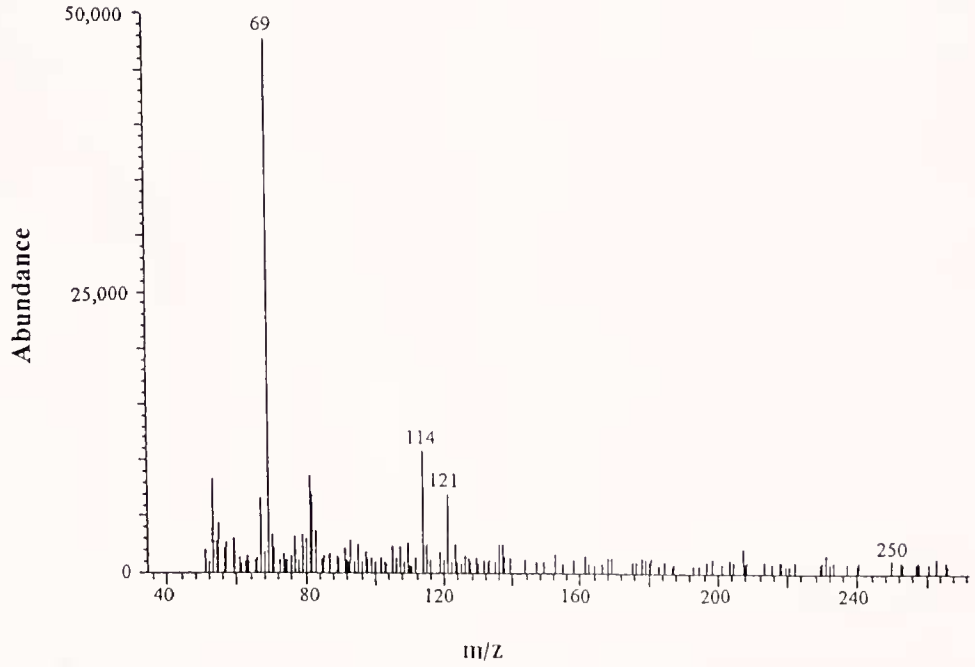

Figure 2. Mass spectrum of methyl farnesoate extracted from *Macrobrachium rosenbergii* larvae. Methyl farnesoate peaks were collected from HPLC separation of larvae extract and analyzed by GC-MS.

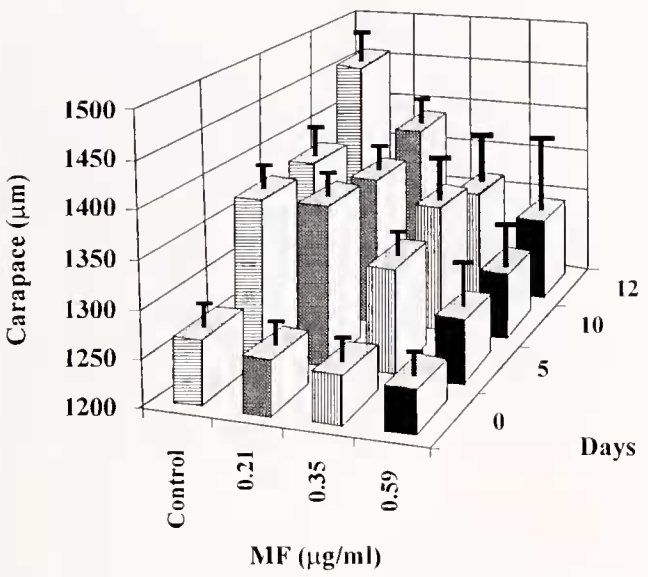

Figure 3. Average carapace length of *Macrobrachium rosenbergii* larvae during 12 days of feeding on *Artemia* enriched with different concentrations of methyl farnesoate. Each bar represents mean ± SE (n = 15). The results presented are taken from one representative experiment out of the three identical experiments performed. Using the *Artemia* vector, approximately 0.0325% of the MF added to the *Artemia* enrichment medium (referred to as MF (μg/ml)) was found in *M. rosenbergii* larva feeding for 8 h on the enriched *Artemia* (Abdu *et al.*, 1998). This suggests that the level of MF in the present study did not exceed 0.191 ng MF per larva exposed to the highest dose and 0.068 ng MF per larva exposed to the lowest dose. Since the average weight of a larva is about 0.002 g, these levels could be expressed as 34–95.5 ng MF/g. The calculated level for the low MF exposure is in the range of physiological level found in *M. rosenbergii* larvae (up to 25 ng/g, data not shown) and adults (up to 40 ng/ml, Sagi *et al.*, 1991). Growth retardation in larvae fed on *Artemia* enriched with the median dose of MF was significant ($P < 0.001$) only on day 12. Growth of larvae fed on *Artemia* that were enriched with the highest concentration of MF was markedly slower than that of the control group, the difference being significant ($P < 0.001$) from day 5 onward.

representative HPLC chromatogram of one such hexane extract of larvae shows the presence of the biological *all-trans* MF, identified by comparing its retention time with that of the external standard (data not shown) and that of the internal standard (Fig. 1). Gas chromatography-mass spectrometry of the putative peaks from larval stages 4, 6, 9, 10, and 11 showed a typical methyl farnesoate profile exhibiting selected ion monitoring at m/z 69, 114, and 121 (Fig. 2).

Effect of methyl farnesoate on late larval growth and development

Survival was similar in the control and all the treatment groups (35% in control, and 47%, 41%, and 46% in the treated groups from the low to the high MF concentration, respectively). An earlier retardation of carapace length was observed in the late larval stages of *M. rosenbergii* exposed to higher doses of MF (Fig. 3). The control group and the larvae fed on *Artemia* that were enriched with the lowest concentration of MF exhibited the fastest growth. Growth retardation in larvae fed on *Artemia* enriched with the median dose of MF was significant ($P < 0.001$) only on day 12. Growth of larvae fed on *Artemia* that were enriched with the highest concentration of MF was markedly slower than that of the control group, the difference being significant ($P < 0.001$) from day 5 onward; after that time, the "high-dose" group almost ceased growing. MF also retarded larval development, as manifested by larval stage (Fig. 4). The control group and the larvae fed on *Artemia* enriched with the lowest concentration of MF developed rapidly, reaching an average larval stage above 10 after 5 days and an average larval stage of above 11 at the

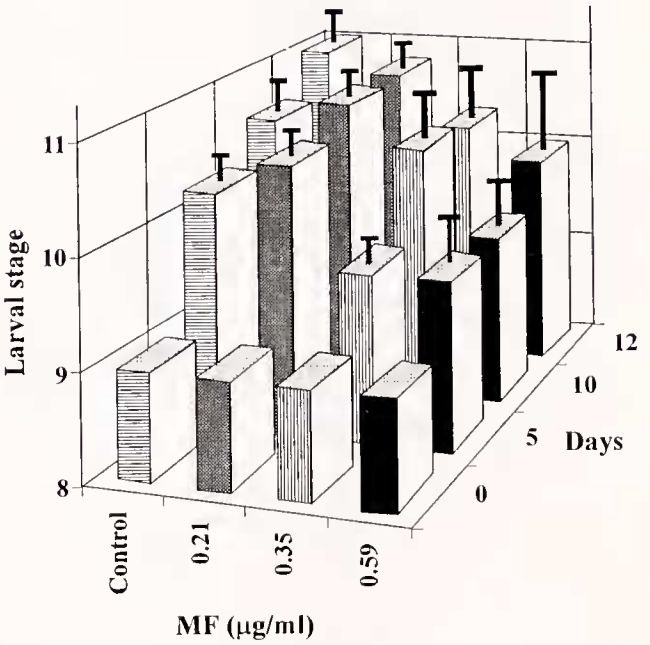

Figure 4. Average larval stage of *Macrobrachium rosenbergii* during 12 days of feeding on *Artemia* enriched with different concentrations of methyl farnesoate. Each bar represents mean ± SE (n = 15). The results presented are taken from one representative experiment out of the three identical experiments performed. MF (μg/ml) refers to the amount of MF added to the medium in which the *Artemia* were incubated prior to being provided as food to *M. rosenbergii* larvae. In the larvae fed on *Artemia* enriched with the median dose of MF, further development was significantly retarded up to the 5th day ($P < 0.001$, *vs.* control group), but not from day 5 to day 10. After day 10, developmental progress was again markedly slower, and on day 12 the average stage of this treatment group was significantly behind that of the control group ($P < 0.001$). Larvae fed on *Artemia* enriched with the highest dose of MF showed significantly slower development to advanced stages than the control group, starting on day 5 ($P < 0.001$); this effect lasted till the end of the experiment.

end of the experiment (after 12 days). In the larvae fed on *Artemia* enriched with the median dose of MF, further development was significantly retarded up to the 5th day ($P < 0.001$, *vs.* control group), but not from day 5 to day 10. After day 10, developmental progress was again markedly slower, and on day 12 the average stage of this treatment group was significantly behind that of the control group ($P < 0.001$). Larvae fed on *Artemia* enriched with the highest dose of MF showed significantly slower development to advanced stages than the control group, starting on day 5 ($P < 0.001$); this effect lasted till the end of the experiment, and only a small progression of larval stages was recorded from that day onward.

Effect of methyl farnesoate on metamorphosis

Metamorphosis—which takes place after the 11th larval stage—was delayed in a dose-dependent manner in larvae treated with MF, but survival was similar to that in the control. In summary, the three experiments showed that among the surviving control individuals, 28 (58%) reached the postlarval stage at the end of the experiment. In the low MF concentration 20 (31%) normal post-larvae were found, and as higher MF concentrations were used, significantly fewer post-larvae were found—only 8 (14%) and 5 (8%), respectively. In addition to the delay in metamorphosis, different forms possessing both larval

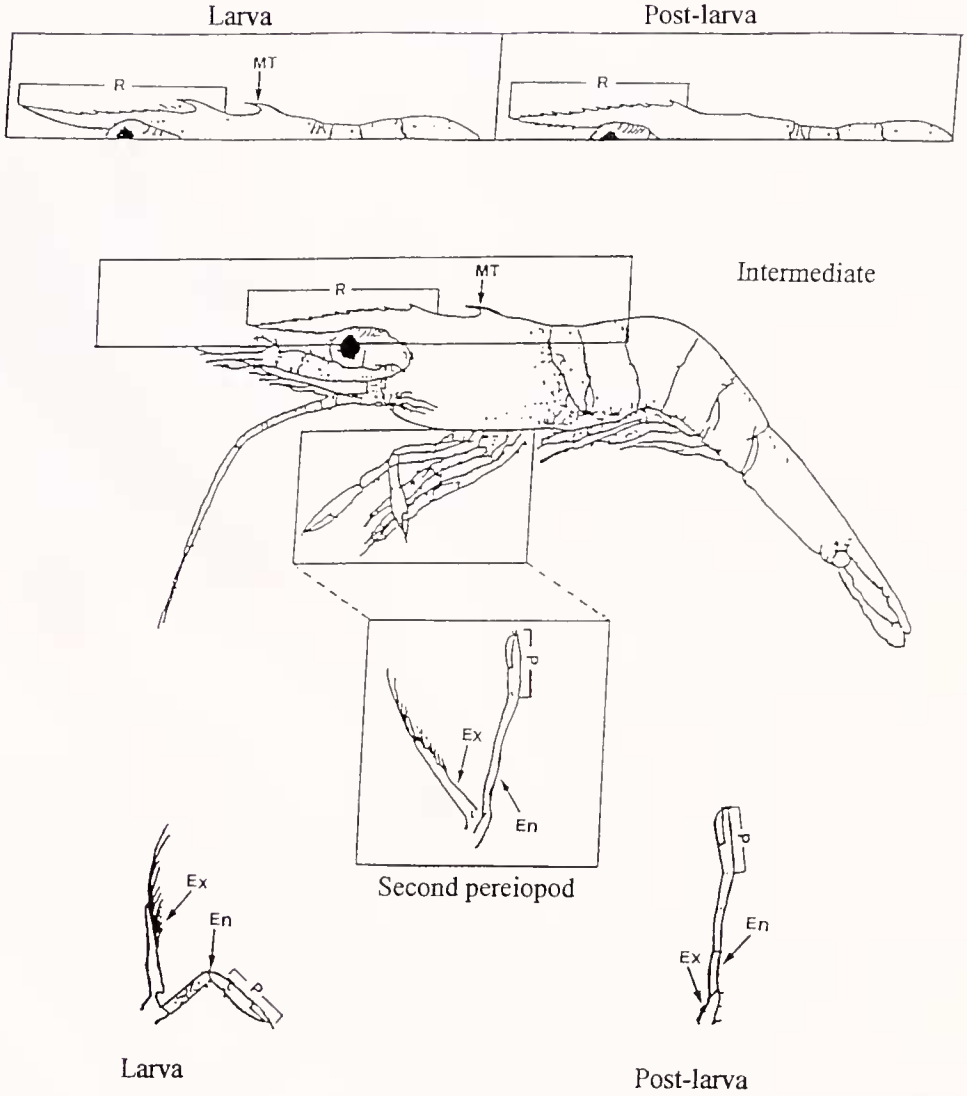

Figure 5. Schematic drawing of larval and postlarval features in a representative intermediate *Macrobrachium rosenbergii*. R - rostrum, MT - median teeth, P - propodus, Ex - exopod of the second pereiopod, En - endopod of the second pereiopod.

	Larvae Stage 11	Intermediate			Post-larvae
		Larval-like	Intermediate	Postlarval-like	
Shape of body	Bent			Straight	
Exopods of pereiopods	Long			Short	
Second pereiopod	Larval	Post larval			
Carapace median tooth	Present				Absent
Rostrum	Larval		Postlarval		
Behavior	Planktonic			Benthic	

Figure 6. Typical morphological and behavioral profiles of the three intermediate types of *Macrobrachium rosenbergii* possessing both larval and postlarval features. The clear and shaded backgrounds represent larval and postlarval features, respectively.

and post-larval features of the six defining characteristics (see Materials and Methods) were observed. Figure 5 illustrates these features in a representative intermediate specimen. At the end of the experiment, three such types were defined; their morphological and behavioral features are summarized in Figure 6. The first type, whose features were mainly larval, was termed "larval-like intermediate" (Fig. 6). It retained five larval features but possessed a postlarval-like second pereiopod (Fig. 5). The second type, which exhibited a more equally divided set of features, was termed "intermediate" (Figs. 5 and 6). It retained three larval features, while possessing a postlarval-like rostrum and second pereiopod (Figs. 5 and 6), and exhibited mixed—partly benthic and partly planktonic—behavior. The third type—termed "postlarval-like intermediate"—retained the larval carapace median tooth but had four of the morphological postlarval features as well as postlarval benthic behavior (Fig. 6).

The relative abundances of the above-described types in the treated and control groups, as well as in a sample from a normal hatchery population, are compared in Figure 7. The postlarval-like intermediate was present in all groups (1%–7%), with no significant difference in relative abundance among the groups. The larval-like intermediate was not found in the source population or in the control group, but it was present in all the MF-treated groups in similar relative abundance (11%–17%). The intermediate type was found only in the two highest MF treatments. In general, the different intermediate types were more significantly abundant ($P < 0.001$) in the two higher MF-treated groups than in the lowest MF treatment group, the normal population, and the untreated control group. The abundance of all three intermediate types increased with the increase in MF concentration (from 2% in the control to 32% in the high MF concentration).

Discussion

A great deal of evidence already supports the idea that MF is a crustacean hormone (Homola and Chang, 1997b), *i.e.*, the existence of a specific endocrine gland (Laufer *et al.*, 1987); specific MF-binding proteins in the circulation (Laufer and Albrecht, 1990; King *et al.*, 1993; Prestwich *et al.*, 1996; Tamone *et al.*, 1997); prompt degradation of MF by specific esterases (King *et al.*, 1993; Homola and Chang, 1997a; Takac *et al.*, 1997); and neuroendocrine regulation of MF secretion (Liu and Laufer, 1996; Wainwright *et al.*, 1996). However, the regulatory importance of MF as a juvenile hormone in the larval development of crustaceans has not been clearly demonstrated, except by circumstantial evidence for its juvenilizing effect on morphogenesis in *Libinia emarginata* (Laufer *et al.*, 1997). The only instances in which such a regulatory role for MF has been suggested are the retardation of metamorphosis reported by Borst *et al.* (1987) in *Homarus americanus* larvae exposed to MF; the retardation of early larval development in *Macrobrachium rosen-*

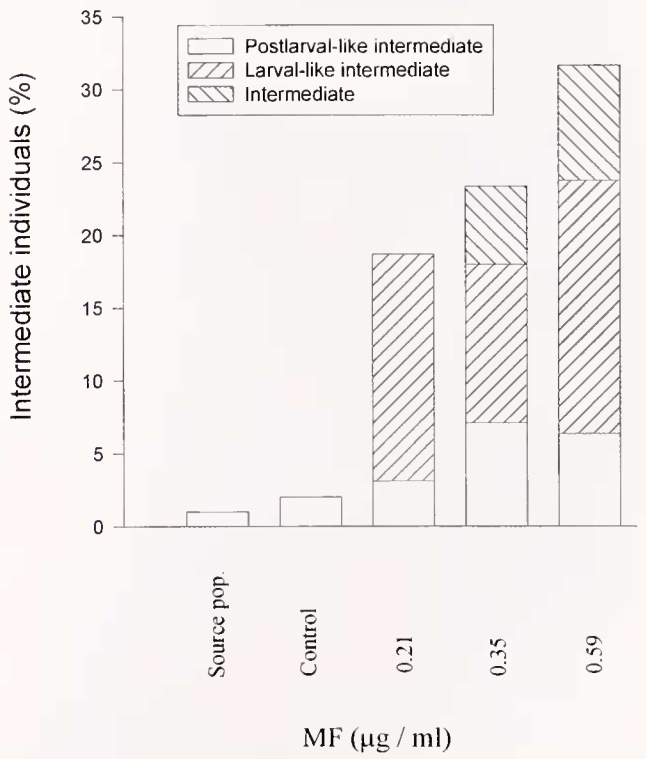

Figure 7. Relative abundance of *Macrobrachium rosenbergii* intermediate types in experimental groups exposed to different doses of methyl farnesoate, in an untreated control group and a sample from a normal hatchery population.

bergii (Abdu, 1996; Abdu *et al.*, 1998); and finally, the findings of the present study in which the retardation of metamorphosis and the appearance of intermediate *M. rosenbergii* types were observed after larvae were exposed to exogenous MF.

In insects, inhibition of metamorphosis is primarily due to the morphogenetic action of a juvenoid. This morphogenetic effect is routinely scored by recording the preservation of juvenile characters after the metamorphic ecdysis. Typically, early treatment of the last-instar larvae induces molting into supernumerary larval instars. Later treatment results in a reduced effect—appearance of intermediates between the juvenile and metamorphosed stages—since the various body tissues gradually become insensitive to juvenoids (for review see Sehnal, 1983). As with insects, the most striking result of this study was the appearance, in the MF-treated groups, of intermediate *M. rosenbergii* individuals. It is not clear how our findings relate to those in insects—whether the different intermediate individuals in the present study correspond to the supernumerary larvae or the intermediate forms known in insects.

The retardation in larval growth found in this study may be due to a molt inhibition, as in some insect species in which continuous exposure to juvenoids can cause a permanent molting block (see Sehnal, 1983, for a review). On the other hand, juvenoids can accelerate the molting process in insects (Sehnal, 1983), and in crustaceans MF may stimulate the production of 20-hydroxyecdysone (Chang *et al.*, 1993); this suggests that in *M. rosenbergii* the retardation may be due to continuous molting to further larval stages, as is the case for the supernumerary larvae in certain insect species (Sehnal, 1983). Further study of the relationship between MF and molting is necessary to determine whether the retardation of growth results from molt inhibition or molt acceleration.

Although the levels of MF administered to the *M. rosenbergii* larvae were within the physiological range (Takac, pers. comm. and Abdu *et al.*, 1998), the possibility of nonspecific toxicity could not be entirely ruled out. In crustaceans, intermediate larval forms may be produced by eyestalk removal during a "critical period," as has been shown for *Rhithropanopeus harrisii* (Costlow, 1966a), *Sesarma reticulatum* (Costlow, 1966b), *H. americanus* (Charmantier and Aiken, 1987), *Palaemonetes varians* (Le Roux, 1984), and *Alpheus heterochaelis* (Knowlton, 1994). Snyder and Chang (1986) suggested that intermediate larval forms in *H. americanus* were probably a manifestation of inadequate nutrition or other stresses. However, Knowlton (1994) showed that the appearance of intermediate forms in *A. heterochaelis* larvae, which have a lecithotropic mode of nutrition, was due to direct or indirect hormonal control from the eyestalk. The reports of the isolation and characterization of mandibular organ-inhibiting hormone (MOIH) from the sinus gland in the eyestalk (Liu and Laufer, 1996; Wainwright *et al.*, 1996), together with the present work, suggest that MF could be the direct hormonal agent controlling metamorphosis in crustaceans through an indirect effect of eyestalk neuropeptides.

MF has recently been described as a "crustacean juvenile hormone in search of functions" (Homola and Chang, 1997b). The findings of the present study—the delay in larval development and metamorphosis in *M. rosenbergii*, together with the increase in the abundance of intermediate specimens with the increase in exogenous MF—contribute to the debate over the physiological function of MF, supporting its possible role as a juvenile hormone in the development and metamorphosis of crustaceans.

Acknowledgments

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